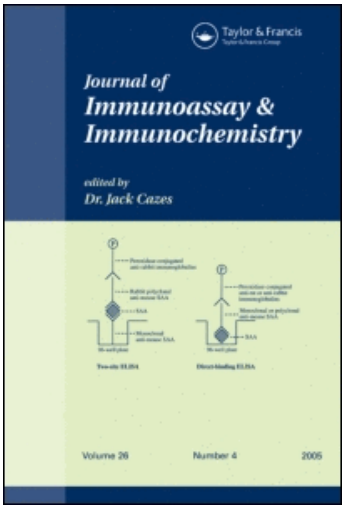

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ENZYME IMMUNOASSAY MEASUREMENT OF THYMOSIN β_4

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ABSTRACT

An enzyme immunoassay for the measurement of thymosin β_4, a chemically characterized thymic polypeptide, is described. A limited amount of reagent antiserum to synthetic thymosin β_4 is incubated with liquid-phase thymosin β_4. Unbound antibody is then incubated with solid-phase thymosin β_4. The method is specific, sensitive, highly reproducible and capable of detecting as little as 2.5 ng/ml of thymosin β_4. (KEY WORDS: Thymic hormones, Thymosin β_4, enzymoimmunoassay)

INTRODUCTION

Thymosin β_4 is a polypeptide isolated from thymosin fraction 5, an extract of calf thymus (1). Thymosin β_4 is one of a family of polypeptides which have been shown to display hormonelike activity. The amino acid sequence of thymosin β_4 has been determined and the synthetic product utilized to assess biological activity. Thymosin β_4 contains 43 amino acid residues, has a molecular weight of 4963 and an isoelectric point of 5.1 (1, 2). It has been shown to induce the expression of terminal deoxynucleotidyl transferase in transferase negative

murine lymphocytes both in vivo and in vitro (3). Thymosin β_4 has also been found to elevate luteinizing hormone releasing hormone and luteinizing hormone in an in vitro sequential hypothalamus-pituitary culture system (4) and after in vivo intracerebral administration (5).

In this study we describe an enzyme immunoassay (EIA) for the measurement of thymosin β_4. It is a sensitive, specific and reproducible assay which utilizes the entire, unaltered thymosin β_4 molecule instead of the analogue (Tyr')-C13-thymosin β_4 required for radioiodination in a previously described radio-immunoassay (RIA) (6).

The EIA approach for measuring thymosin β_4 has potential use for determining serum levels in patients with suspected primary or secondary immunodeficiency disorders and in patients with suspected reproductive abnormalities (7).

MATERIALS AND METHODS

Synthetic thymosin β_4 was custom synthesized by Peninsula Laboratories, Incorporated, Belmont, CA. Antiserum to thymosin β_4 was prepared as previously described (6). These reagents were generously supplied by Alpha 1 Biomedicals, Washington, D.C. Goat anti-rabbit IgG (GARG) was obtained from Cooper Biomedical, Inc., Malvern, PA (lot #24329). Protein A conjugated to alkaline phosphatase (lot #31222) was obtained from Zymed Laboratories, Burlingame, CA. Protein A binds to both GARG and the antibody to thymosin β_4, increasing the sensitivity of the enzyme conjugate reaction. All antibody and antigen reagents were aliquoted

in stock solutions and stored frozen until use, with the exception of the enzyme conjugate, which was refrigerated.

A stock solution of synthetic thymosin β_4 prepared in phosphate buffered saline at a concentration suitable for the working range of the assay, i.e., 6 dose levels between 2.5 and 80 ng/ml, was aliquoted and stored at -20°C until use for preparation of the standard curve. The EIA buffer was phosphate buffered saline: 8.0 gm of NaCl, 0.2 gm of KH_2PO_4, 1.15 gm of Na_2HPO_4 and 0.2 gm of KCl to which was added 0.75 ml of Tween 20 and 0.2 gm of NaN_3 in 1 liter of distilled water pH 7.4 (PTA).

Standard solutions of synthetic thymosin β_4 (500 µl) were added to 12 X 75 mm minisorp tubes (Nunc Immunotest tubes). Then 500 µl of a 1:5000 dilution of stock antiserum was added to each tube. The tubes were vortexed, sealed with parafilm and incubated overnight at 4°C. Wells of an Immulon 2, flat bottom microtiter plate (Dynatech Laboratories, Alexandria, VA) were coated with synthetic thymosin β_4 by adding 200 µl/well of a 50 ng/ml thymosin β_4 solution in Dulbecco's phosphate buffered saline (Gibco, Grand Island, NY), and incubated overnight at 4°C. The plate was washed with PTA and 200 µl/well of each antibody-antigen solution was added to three coated and one uncoated well and incubated overnight at 4°C. The plate was washed with PTA and a 1:2000 dilution of GARG (0.5 µg/ml; 200 µl/well) was added to the plate and incubated for 1 h at room temperature. The plate was washed with PTA and a 1:2000 dilution of the enzyme conjugate (200 µl/well) was added to the plate and incubated for

2 h at room temperature. The plate was washed with PTA and 200 µl/well of 1 mg/ml of substrate (p-nitrophenyl phosphate, disodium; Sigma Chemical Co., St. Louis, MO) was added in carbonate-bicarbonate buffer pH 9.8. Optical density (OD) readings were taken at 25, 33 1/3, 50 and 100 minutes using a Titertek Multiskan Spectrophotometer (Flow Laboratories, McLean, VA). All absorbance values reported were obtained at 405 nm and calculated to 100 minutes of development. Values above 1.0 were extrapolated linearly from the time at which the reading was approximately 1.0. All tests were done in quadruplicate with the absorbance of the uncoated well subtracted from the average of three coated wells.

RESULTS

Coating Concentration Kinetics

Table 1 gives the absorbance readings obtained after 100 minutes of development in a plate coated with six concentrations of thymosin β_4 and using three dilutions (1:2000, 1:5000 and 1:10000) of the primary antibody. The plate was developed as previously described. The amount of primary antibody bound was directly dependent on the combination of the coating concentration and the primary antibody dilution. The kinetics were consistent utilizing any of the primary antibody dilutions and approached a peak absorbance with each dilution at a coating concentration of 100 ng/ml. We observed that a "high" coating concentration combined with a "low" primary antibody concentration yielding a

TABLE 1

Optimal Coating Concentration

[Thymosin β_4][a]	Primary Antibody Dilution					
	1:2000		1:5000		1:10000	
	OD[b]	BKG[c]	OD	BKG	OD	BKG
1 ng/ml	0.426	0.020	0.198	0.012	0.123	0.006
5 ng/ml	2.474	0.026	1.802	0.008	1.195	0.006
10 ng/ml	3.297	0.008	2.481	0.014	1.779	0.000
25 ng/ml	4.094	0.020	3.151	0.018	2.281	0.004
50 ng/ml	4.441	0.022	3.362	0.012	2.506	0.004
100 ng/ml	4.642	0.018	3.529	0.008	2.673	0.000

[a]Concentration of thymosin β_4 in coating solution added to wells.

[b]Absorbance at 405 nm after reaction for 100 min in wells coated with thymosin β_4.

[c]Absorbance at 405 nm after reaction for 100 min in uncoated wells.

peak OD between 2.0 and 3.0 was most suitable for developing the standard curve. Based on that criterion, a coating of 50 ng/ml of thymosin β_4 and a final primary antibody concentration of 1:10000 were used in the subsequent experiments.

Primary Antibody Binding

The rate at which the primary antibody binds to the coating in the wells and the effects of concentration and temperature on the kinetics of binding are shown in Figures 1 and 2. Three

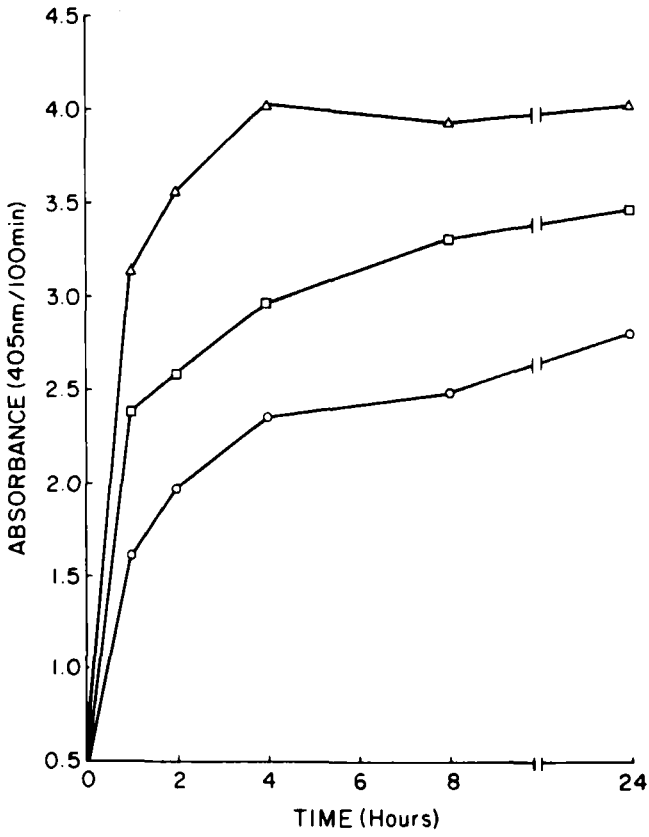

FIGURE 1. Kinetics of primary antibody binding at 22°C. Wells were coated with 50 ng/ml thymosin β_4. Antiserum to thymosin β_4 was added at dilutions of 1:2000 (△), 1:5000 (□) and 1:10000 (○) for the interval indicated. This was followed by reaction with GARG (2 X 10^{-3} dilution) and then enzyme conjugate (2 X 10^{-3} dilution).

dilutions of primary antibody were used (1:2000, 1:5000 and 1:10000) to demonstrate the binding kinetics over time. Figure 1 shows the binding kinetics at room temperature (22°C) and figure 2 shows the kinetics at 4°C. The plates were coated with 50 ng/ml of thymosin β_4, then primary antibody was added and allowed to

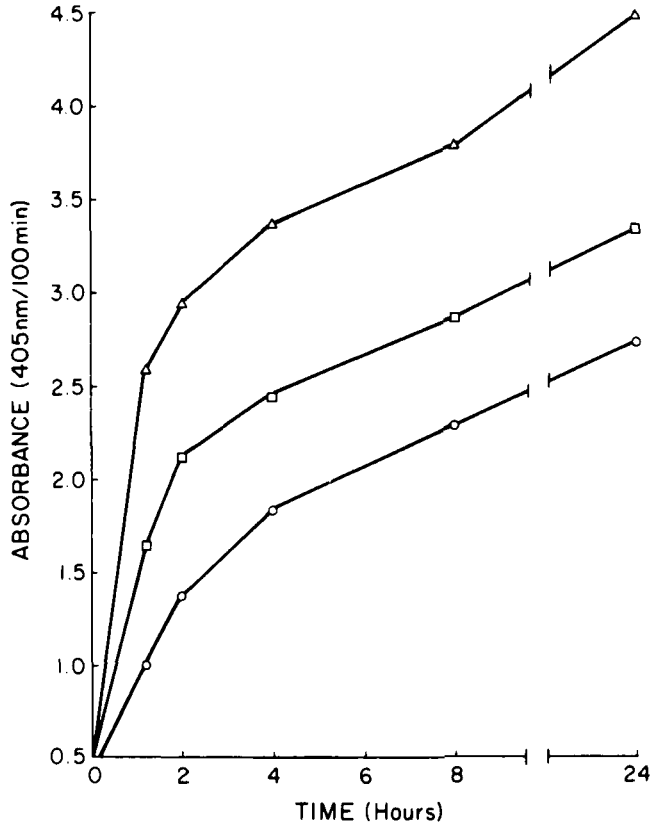

FIGURE 2. Kinetics of primary antibody binding at 4°C. Wells were coated with 50 ng/ml thymosin β_4. Antiserum to thymosin β_4 was added at dilutions of 1:2000 (△), 1:5000 (□) and 1:10000 (○) for the interval indicated. This was followed by reaction with GARG (2 X 10^{-3} dilution) and then enzyme conjugate (2 X 10^{-3} dilution).

incubate for 1, 2, 4, 8, 16 and 24 h. The secondary antibody was added at a 1:2000 dilution for 1 h and enzyme conjugate at a 1:2000 dilution for 2 h. The plate was developed as described. The rate of binding of the primary antibody to the antigen at 22°C was initially greater than the rate at 4°C until 16 h but

reached similar peaks after 24 h. The 1:10000 dilution of the primary antibody exhibited the smoothest binding characteristics at both temperatures.

Secondary Antibody Binding

Figure 3 shows the rate of binding of the secondary antibody to the primary antibody and the effect of concentration on the kinetics. The plates were coated as described, then a 1:10000 dilution of primary antibody was added for 22 h at 4°C. Three dilutions of the secondary antibody (1:1000, 1:2000 and 1:4000) were added and allowed to incubate for 1/2, 1, 2 and 4 h at room temperature. Enzyme conjugate was added for 2 h and the plates were developed as described. The reaction was complete at 2 h with little change when extended to 4 h. A 1:2000 dilution gave adequate binding with minimal background.

Enzyme Conjugate Binding

The rate of binding of the enzyme conjugate to the primary-secondary antibody complex is shown in Figure 4. Three dilutions of enzyme conjugate were used (1:1000, 1:2000 and 1:4000) to determine the effect of concentration on the kinetics. Plates were prepared with coating, primary antibody and secondary antibody as described, then enzyme conjugate was added and allowed to incubate 1/2, 1, 2 and 4 h at room temperature. The binding kinetics were very consistent at all dilutions with adequate binding and minimal background obtained after 2 h of incubation with the 1:2000 dilution.

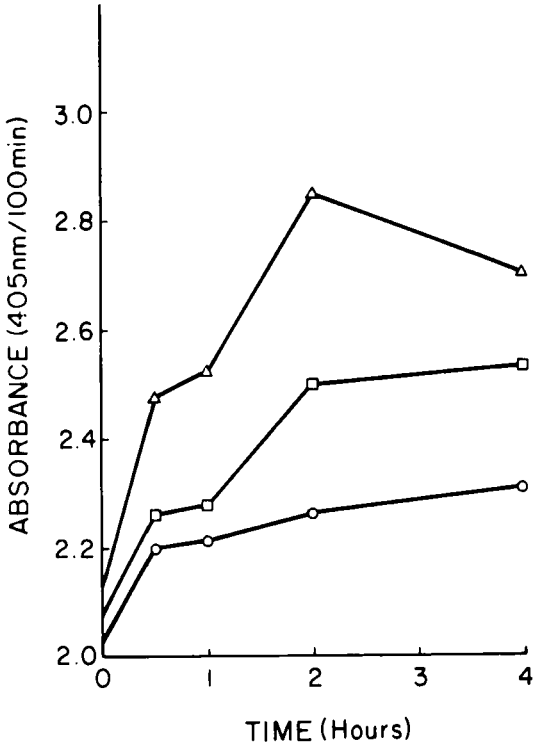

FIGURE 3. Binding kinetics of secondary antibody (GARG). Wells were coated with 50 ng/ml thymosin β_4 and reacted with antiserum to thymosin β_4 (1:10000) for 22 h at 4°C. This was followed by the addition of GARG at a dilution of 1:1000 (△), 1:2000 (□) or 1:4000 (○) for the interval indicated with subsequent reaction using enzyme conjugate (1:2000 dilution).

Dose-Response of the Synthetic Standard

Synthetic thymosin β_4 produced a dose-response relationship as shown in Table 2. The EIA exhibited the characteristics of having a minimal detectable dose of 2.5 ng/ml and a useful operating range between 2.5 and 80 ng/ml. Regression analysis of the concentration of synthetic thymosin β_4 versus absorbance on 10 different standard curves gave a mean slope of -0.728 ± 0.033

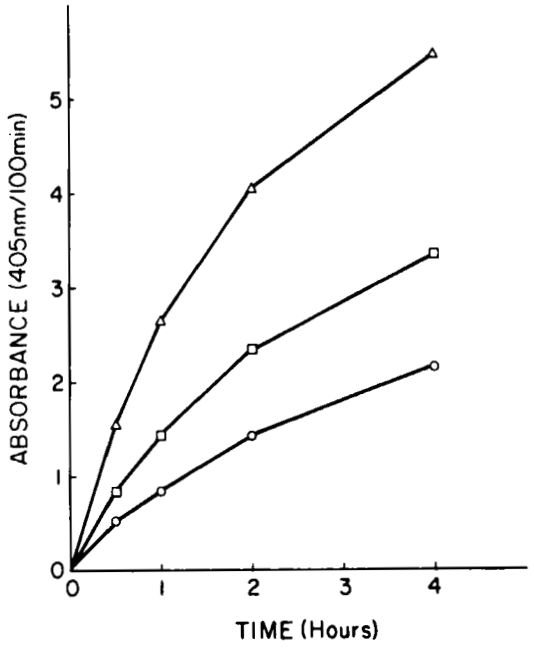

FIGURE 4. Enzyme conjugate reaction. Wells were coated with 50 ng/ml thymosin β_4 and reacted with antiserum to thymosin β_4 (1:10000 dilution) for 22 h at 4°C. This was followed by reaction with GARG (1:2000 dilution) for 2 h. Three dilutions of enzyme conjugate were added at 1:1000 (△), 1:2000 (□) or 1:4000 (○) for the interval indicated at room temperature.

(SD), a mean y-intercept of 2.187 ± 0.122 and a mean r value of 0.992 ± 0.004. The variation within assays averaged 3.1%, between assays 9.5%. The slope and the y-intercept are effected by the incubation times required in the assay, particularly that for the enzyme conjugate. The observed deviations reported between assays may reflect minor fluctuations in incubation times.

Specificity of the EIA

The specificity of the EIA was determined by assaying preparations of the putative thymic hormone, thymic humoral

TABLE 2

Standard Dose-Response Curve for Synthetic Thymosin β_4

[Thymosin β_4][a]	Within-assay Variation			Between-assay Variation		
	n	Mean ± SD[b]	CV[c]	n	Mean ± SD	CV
0	6	2.186 ± 0.047	2.2	5	2.168 ± 0.198	9.1
2.5 ng/ml	6	1.833 ± 0.093	5.1	5	1.813 ± 0.168	9.3
5 ng/ml	6	1.729 ± 0.043	2.5	5	1.691 ± 0.117	6.9
10 ng/ml	6	1.516 ± 0.047	3.1	5	1.482 ± 0.135	9.1
20 ng/ml	6	1.312 ± 0.029	2.2	5	1.266 ± 0.119	9.4
40 ng/ml	6	1.019 ± 0.035	3.4	5	1.032 ± 0.108	10.5
80 ng/ml	6	0.878 ± 0.027	3.1	5	0.801 ± 0.099	12.4

[a] PTA containing increasing concentrations of liquid-phase thymosin β_4 and a constant dilution of antiserum to thymosin β_4 (1:10000) was allowed to react in wells for 22 h at 4° C.

[b] Absorbance at 405 nm after reaction for 100 min.

[c] CV, percentage coefficient of variance.

factor (THF) and other serum proteins as shown in Table 3. Increasing amounts of each preparation were added to the EIA system until levels were reached which were well above the known physiological concentrations in serum or until a practical limit was reached. For those preparations which did not produce a response significantly different from the zero dose level, the largest dose tested is indicated. Thymosin β_4 or cross reacting material was detected in THF and quantitated at 65 ng/mg of THF. Thymosin fraction 5 and spleen fraction 5 contained detectable levels of thymosin β_4 which became measurable at 100 μg/ml.

TABLE 3

Specificity of EIA for Thymosin β_4

Proteins	Amount required to generate a 20% drop in the absorbance.
A. Non-Thymus	
Insulin	>10 µg/ml[a]
Glucagon	>10 µg/ml
Albumin (human)	>10 mg/ml
Albumin (bovine)	>10 mg/ml
B. Thymus-related	
Thymosin fraction 5	100 µg/ml
Spleen fraction 5	100 µg/ml
Thymosin α_1	>1 µg/ml
Thymic humoral factor	50 µg/ml

[a]The values reported for insulin, glucagon, human albumin, bovine albumin and thymosin α_1 represent the highest concentration of the proteins tested and which did not effect absorbance as compared to zero dose level.

There was no cross-reactivity detected with any of the serum proteins tested.

DISCUSSION

The development of the EIA was made possible by the binding affinity and stability of the primary antibody-antigen complex at 4° C. The EIA for thymosin β_4 was developed as an alternative to

the RIA. The primary antibody used in the development of the EIA is identical to that used in the previously described RIA (6) and has been shown not to exhibit cross-reactivity with common serum proteins and protein hormones. The synthetic thymosin β_4 standard produced a dose response which was linear and useful for quantitation in a range from 2.5 to 80 ng/ml. The binding kinetics of all the reagents are very stable and predictable resulting in excellent reproducibility of the assay.

In the development of the EIA it was determined that there were two distinct approaches that could be used to generate a standard curve based on the kinetics of the primary antibody binding. The difference between these methods resides in the temperature at which the primary antibody is incubated with the varying concentrations of thymosin β_4 in the tubes. The incubation temperature may be at 4°C overnight (18-24 h), which we term the long method; or at room temperature for 4 h, which we term the short method. In both instances the liquid phase thymosin β_4 primary antibody solutions are then added to the coated plate and incubated overnight (18-24 h) at 4°C. These incubation conditions are critical for generating a smooth curve. The slopes and *y*-intercepts obtained using these two methods were slightly different so it remains to be determined which will be more suitable for serum measurements. It was also determined that the plate could be adequately coated in 4 h at room temperature, therefore the short method reduces the time to complete the entire assay by one day. The variability and

specificity data reported here were obtained using the long method.

There are other alterations which could be made in the methods described herein. The use of GARG is not essential for the development of the curve but it does significantly enhance the resolution of the curve. The incubation time of the GARG and enzyme conjugate can be altered to change the rate of development of the substrate. However increasing the incubation time increases the background, although not significantly. Any of these changes effect the slope and y-intercept of the curve but do not appear to effect the intrinsic accuracy of the assay.

The aforementioned alterations permit considerable flexibility when running the assay to accommodate for different schedules and equipment demands. The EIA is a viable and perhaps preferable alternative to the RIA since no isotopes are utilized and the synthetic analog [^{125} I(Tyr')-C13] of the naturally occurring thymosin β_4 is not required. The EIA utilizes the entire 43 amino acid molecule identical to that which is in serum. This could conceivably result in more accurate measurements of serum levels.

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